COMPLEMENT FIXATION WITH PROTEIN SUBSTANCES

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REUBEN L. KAHN, B.S., M.S.

A THESIS

Submitted to the Faculty of New York University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Science
in the Department of Bacteriology and Hygiene

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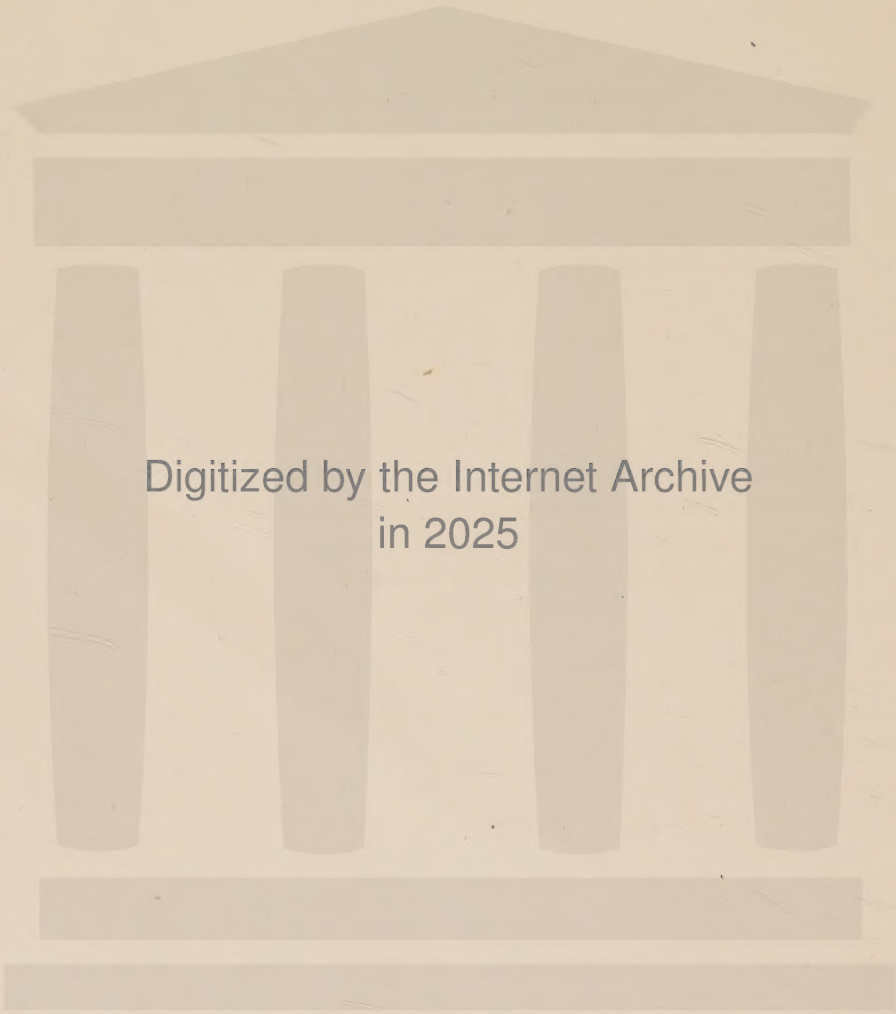
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COMPLEMENT FIXATION WITH PROTEIN SUBSTANCES

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The classical studies of Wells and Osborne on the biological reaction of vegetable proteins (1) have demonstrated that the specificity of the anaphylactic reaction depends upon the chemical structure of the protein molecule, and if the molecule be split—whether by acid hydrolysis or peptic digestion—its anaphylactic specificity is lost.

Whether or not these findings are applicable also to the complement fixation reaction is the aim of the introductory studies recorded below. The substances employed consisted of proteins, split proteins, racemized proteins, and animal tissues. These substances were introduced parenterally into rabbits and specific complement fixing antibodies sought for in the blood of these animals at definite intervals. Knowing the chemical nature of the immunizing substance, we were thus able to judge whether proteins of various origin and chemical constitution are able to call forth in the animal organism, specific complement fixing antibodies, and if so, to what degree a protein molecule may be modified or split and still retain this property.

INTRODUCTION

Complement fixation with purified proteins have been reported by several investigators. Dunbar (2) obtained specific complement fixing sera on injection of pollen from various plants. White and Avery (3) demonstrated the presence of complement-fixing antibodies in the serum of rabbits immunized with edestin. More

recently, Lake, Osborne and Wells (4) have further established the fact that purified vegetable proteins call forth the production of specific complement-fixing antibodies.

In our studies, two vegetable proteins of high purity—edestin from hempseed and phaseolin from kidney bean—and three animal proteins of relative purity—casein, lactalbumin and gelatin—were employed. Casein and lactalbumin are found side by side in milk, and it seemed worth while to see whether these substances could be interchanged as antigens. Gelatin was used because of its peculiar chemical construction. This protein, as is well known, lacks cystine, tyrosin and tryptophane and from the standpoint of nutrition it behaves somewhat like a carbohydrate. Furthermore Vaughan (5) has been unable to obtain a soluble toxin from it and according to Wells (6) it does not produce anaphylactic antibodies. Whether or not, therefore, it will call forth complement-fixing antibodies is of considerable interest.

When turning to the question of the production of antibodies on injections with split proteins, one encounters a field relatively unexplored. According to Krause (7), split protein products have not the power to produce specific precipitin antibodies. Wells (8) investigated the different fractions obtained from tryptic and peptic digestion of egg white with a view of calling forth anaphylactic antibodies in guinea-pigs. His findings indicate that the proteoses and peptones as well as the lower products of hydrolysis obtained from this protein, could not sensitize guinea-pigs, either with egg white or the digestive products themselves. This investigator concludes that as far as anaphylaxis is concerned, nothing less than the entire protein molecule can act as an antigen.

The same conclusion is reached in the later studies of Wells and Osborne (9). On the other hand, proteoses from various seeds, according to these investigators, produce strong anaphylactic reactions in animals. The explanation being that these so-called proteoses of vegetable origin are not in any way similar to products of peptic digestion or acid hydrolysis of proteins. In fact these vegetable proteoses are chemically similar to soluble

animal proteins, which explains their high anaphylactic specificity.

We were unable to find a record of complement fixation studies with split protein products. It seemed therefore worth while to investigate the question whether the immunological behavior of such substances in so far as the complement fixation reaction is concerned, would be similar to that of the precipitin and anaphylactic reactions.

The following split proteins were employed in this series: (1) Proteose from acid digestion of racemized casein; (2) Proteose from gliadin by acid digestion; (3) Proteose formed during the preparation of racemized zein; (4) Proteose formed during the racemization of edestin; (5) Vaughan's crude soluble poison from casein, and (6) Insoluble residue remaining from preparation of Vaughan's crude soluble poison from casein.

Racemized proteins also furnish good material for immunological study. These modified proteins, it will be recalled, are prepared by digesting protein in large quantities of normal sodium hydroxide and permitting the mixture to stand for a long interval, during which the plane of polarization of the protein is changed. Ten Broeck (10) has investigated the immunological properties of racemized egg albumin in rabbits and guinea-pigs and obtained negative results. Underhill and Hendrix (11), in their physiological studies of these proteins, found that purified racemized proteins show no poisonous action. It seemed worth while to add these modified proteins to our series with the view of determining whether they have the power to call forth complement-fixing antibodies when injected in rabbits. The following racemized proteins were employed; racemized zein, edestin casein, and egg albumin.

Finally, it was desired to obtain some data with regard to the specificity of animal tissues; the question being, will liver tissue or muscle tissue injected into rabbits call forth the production of specific complement-fixing antibodies? In other words, will the immunological behavior of animal tissues be similar to that of blood, the specificity of which is well established? Guinea-pig's muscle and heart tissue were employed in one case with a

view of determining whether rabbits will produce specific antibodies against these and whether heart and skeletal muscle may be interchanged as antigens. In another series, rabbit muscle and liver were employed; this with a view of determining whether an animal's own tissue may act as a foreign antigen. This problem was investigated by Bradley and Sansum (12) by means of the anaphylactic reaction. These investigators found a low order of sensitization on injecting extracts of guinea-pig tissues in guinea-pigs. But, as is well known, weak and "border-line"—anaphylactic reactions present many difficulties in the interpretation of results. It was hoped therefore that the complement fixation reaction might furnish a better medium for the study of the specificity of tissues.

EXPERIMENTAL

Varying modes of immunization for the study of antibody production are reported by different investigators. White and Avery (3) in their immunity studies with edestin injected in a rabbit, 50, 100, 50 and 150 mgm. of edestin at intervals of five, six, and one days respectively, bleeding the animal 19 days later. Lake, Wells and Osborne (4) it appears, observed no hard and fast rule with regards to the method of immunization. In most cases they employed about 3 grams of protein; fractions of which they injected at varying intervals and extended their entire period of immunization from about five to six weeks.

It seemed important, in attempting immunity studies with a variety of substances of different chemical nature, that some standard method be adhered to. It was soon found that 5 injections at 48-hour intervals and final bleeding six days after last injection, was ample time for the production of complement-fixing antibodies. The quantities of protein employed were 50, 75, 100, 125 and 150 mgm., respectively. Later, a still simpler method was employed in a number of cases, giving three injections at twenty-four hour intervals and bleeding eight days after the last injection. The quantity of protein employed in this case being 50, 75, and 100 mgm., respectively.

Table 1 gives an outline of the two methods of immunization employed. Both of these methods produced complement-fixing antibodies in rabbits with purified proteins, and they will be referred to in the text as method 1 and method 2.

Of the proteins employed in immunization, edestin and phaseolin were kindly furnished to us by Dr. Thomas B. Osborne; the casein was a Kahlbaum product; the lactalbumin, an "Arlco" preparation, and the gelatin was of the type generally employed in bacteriological work. The racemized proteins and proteoses, as well as Vaughan's crude soluble poison from casein, and the insoluble residue remaining from its preparation, were kindly furnished to us by Prof. Frank P. Underhill. The preparation of these protein derivatives is given by Underhill and Hendrix

TABLE 1

IMMUNIZATION METHOD NO. 1 INJECTIONS GIVEN AT 48-HOUR INTERVALS			IMMUNIZATION METHOD NO. 2 INJECTIONS GIVEN AT 24 HOUR INTERVALS		
Number of injections	Quantity of protein injected	Time of bleeding	Number of injections	Quantity of protein injected	Time of bleeding
	mgm.			*mgm.*	
First.....	50	Six days after	First.....	50	Eight days after
Second.....	75	last injection	Second.....	75	last injection
Third.....	100		Third.....	100	
Fourth.....	125				
Fifth.....	150				

(13) in their recent studies on the physiological action of these products.

The guinea-pig's muscle and heart and the rabbit's liver and muscle were prepared in the following manner: After bleeding the animals very nearly to completion the tissues were removed, freed from connective tissue, and washed in distilled water. They were then placed in about ten times their quantity in water, slightly acidified with acetic acid. This was done in order to coagulate the proteins and remove the extractives. After ten minutes boiling, the tissues were placed in fresh water and again boiled for ten minutes. This process was repeated three times. After the last boiling, the tissues were dried at 40°C. and ground into a powder form, when they were ready for use.

The injections were made intravenously with the exception of a few instances where intraperitoneal injections were resorted to. Those of the proteins or protein derivatives which are insoluble in water, were rendered soluble just before the injection by the addition of a few drops of normal sodium hydroxide.

Before beginning the injections the sera of the experimental animals were, in a number of cases, tested for complement-fixing antibodies, the proteins intended for injections being used as antigens. Kolmer and Trist (14) recently reported a large number of cases where normal rabbits gave positive complement-fixation reactions with lipoidal and bacterial antigens, without previous immunization with these substances. In no case, however, did we find non-specific binding with the proteins and protein derivatives employed in these studies.

The bleeding was performed under anesthesia and the sera were obtained—after the blood had been permitted to remain over night in the ice-box—practically free from corpuscles.

In table 2 is recorded the kind and quantity of protein injected, also the dates of the injections and bleedings.

The complement-fixation test as it is employed in the Laboratories of the Health Department of New York City was adopted in this work. The following is a description of the method in detail:

Complement. The complement employed was fresh guinea-pigs serum obtained in the following manner: The pig was rendered unconscious by a moderate blow at the junction of the brain and cord. The blood was then obtained by incising with pointed scissors the anterior chest wall directly into the heart. After permitting the blood to clot at room temperature, it was placed in the ice box over night; the serum collected and mixed with sterile saline in the proportion of 1–10. This 10 per cent solution in saline served as complement, and it was freshly prepared for each series of experiments.

Sheep's cells. The blood employed was obtained from the jugular vein of sheep, collected in sterile bottles containing glass beads. After defibrination by shaking, it was freed from serum in the following manner: In a 15-cc. graduated centrifuge tube, the blood was poured to the 8 cc. mark, and made up to about 15 cc. with saline and mixed.

TABLE 2

Protocol of immunization

NUMBER OF EXPERIMENT	DATE OF INJECTIONS	TYPE OF PROTEIN AND QUANTITY INJECTED	NUMBER OF EXPERIMENT	TYPE OF PROTEIN AND QUANTITY INJECTED	DATE OF BLEEDING
		Phaseolin (rabbit A)		Edestin (rabbit B)	
	1915	*mgm.*		*mgm.*	*1915*
1	October 26	50	2	50	
	October 28	75		75	
	October 30	100		100	November 9
	November 1	125		125	
	November 3	150		150	
		Casein (rabbit C)		Lactalbumin (rabbit D)	
3	December 9	5	4	5	
	December 11	5		5	
	December 13	5		5	December 23
	December 15	5		5	
	December 17	5		5	
		Lactalbumin (rabbit E)		Casein (rabbit F)	
	1916				*1916*
5	January 4	50	6	50	
	January 6	75		75	
	January 8	100		100	January 18
	January 10	125		125	
	January 12	150		150	
		Guinea-pig heart (rabbit G)		Guinea-pig muscle (rabbit H)	
7	January 4	50	8	50	
	January 6	75		75	
	January 8	100		100	January 19
	January 10	125		125	
	January 12	150		150	
		Vaughan's soluble poison (rabbit I)		Insoluble residue from Vaughan's poison (rabbit J)	
9	January 25	50	10	50	
	January 27	75		75	
	January 29	100		100	February 8
	January 31	125		125	
	February 2	150		150	

TABLE 2—*Concluded*

NUMBER OF EXPERIMENT	DATE OF INJECTIONS	TYPE OF PROTEIN AND QUANTITY INJECTED	NUMBER OF EXPERIMENT	TYPE OF PROTEIN AND QUANTITY INJECTED	DATE OF BLEEDING
		Proteose from digestion of racemized casein (rabbit K)		Proteose from gliadin by acid digestion (rabbit L)	
	1916	*mgm.*		*mgm.*	*1916*
11	February 29	50	12	50	
	March 1	75		75	March 11
	March 2	100		100	
		Proteose from preparation of racemized zein (rabbit M)		Proteose from preparation of racemized edestin (rabbit N)	
13	February 29	50	14	50	
	March 1	75		75	March 11
	March 2	100		100	
		Racemized zein (rabbit O)		Racemized edestin (rabbit P)	
15	February 29	50	16	50	
	March 2	75		75	
	March 4	100		100	March 14
	March 6	125		125	
	March 8	150		150	
		Rabbit liver (rabbit Q)		Rabbit muscle (rabbit R)	
17	March 4	50	18	50	
	March 6	75		75	
	March 8	100		100	March 18
	March 10	125		125	
	March 12	150		150	
		Racemized casein (rabbit S)		Racemized egg albumin (rabbit T)	
19	March 11	50	20	50	
	March 12	75		75	March 21
	March 13	100		100	
		Phaseolin (rabbit U)		Edestin (rabbit V)	
21	March 30	50	22	50	
	March 31	75		75	April 9
	April 1	100		100	
		Gelatin (rabbit W)			
23	March 30	50			
	March 31	75			April 9
	April 1	100			

This was centrifuged for about five minutes at 2500 revolutions. Clear fluid was now pipetted off, fresh saline added, and again centrifuged as above This wa hing was repeated five times (fresh saline being added each time). The sixth or final washing wa carried out by centrifuging for ten minutes at 2500 revolutions. The amount of packed cells in the graduated centrifuge tube was now read, and the sediment was made up into a 5 per cent suspension in saline. This 5 per cent suspension, also, was freshly prepared for each series of determinations.

Amboceptor. Antisheep's cells amboceptor was employed throughout the work and was obtained by immunizing rabbits with sheep cells. After thoroughly washing the cells in the manner stated above, 50 per cent suspensions in saline were made up and injected intravenously in rabbits at 48 hour intervals. The following quantities were used in the immunization:

First injection 0.5 cc. of 50 per cent suspension of washed sheep cells.
Second injection 1.0 cc. of 50 per cent suspension of washed sheep cells.
Third injection 1.5 cc. of 50 per cent suspension of washed sheep cells.
Fourth injection 2.0 cc. of 50 per cent suspension of washed sheep cells.
Fifth injection 3.0 cc. of 50 per cent suspension of washed sheep cells.

Rabbits were bled six days after the last injection to test the strength of the amboceptor. If a 1–1000 dilution in saline brought about complete hemolysis of the red cells in the presence of complement during one hour at 37°C. it was considered sufficiently strong to be used in our hemolytic system. Rabbits were then completely bled under ether anasthesia. Blood was obtained under sterile precautions by inserting pointed scissors directly into the heart. Serum was removed after complete clotting and, inactivated at 56°C. for thirty minutes for three successive days. Stock solutions of 1–100 in saline were then prepared, and 1–1000 dilutions were freshly prepared from the stock solutions for each series of tests.

Antigen. One hundred milligrams of the protein material in 100 cc. of saline served as the antigen. This was either a solution or a suspension depending on whether the protein substance employed was soluble or insoluble.

Standardization of the hemolytic system. Freshly prepared 5 per cent suspension of sheep's cells, 10 per cent guinea-pigs complement, and 1–1000 amboceptor were placed in a series of 11 Wassermann tubes in the following amounts:

Tube 1 contained 0.1 cc. amboceptor, 0.1 cc. complement, 0.1 cc. sheep's cells.

Tube 2 contained 0.09 cc. amboceptor, 0.1 cc. complement, 0.1 cc. sheep's cells.

Each succeeding tube contained the same amount of complement and sheep's cells, but the quantity of amboceptor decreased by 0.01 cc. in each case. Thus, the tenth tube contained 0.1 cc. complement, 0.1 cc. red cells, and only 0.01 cc. amboceptor.

The eleventh control tube contained complement and cells only, as a test for natural amboceptor in the guinea-pig's serum.

The total volume of each tube was made up to 0.3 cc. with saline and the rack of tubes was placed in the water bath at 37°C. for one hour. The smallest amount of amboceptor that caused complete hemolysis at the end of one hour was considered the unit for that system. If, for example, the tube containing 0.05 cc. of amboceptor was completely hemolyzed at the end of that time, 0.05 cc. was the unit and three times this quantity; i.e., 0.15 cc. of amboceptor was used to sensitize each 0.1 cc. of sheep's cells. Experience has shown that optimum results are obtained by using three units of amboceptor.

Antigen titration. These titrations were carried out in a series of 12 Wassermann tubes. The first six of these tubes contained 0.01 cc. of immune rabbit serum, 0.1 cc. of complement, and antigen in the following proportions: First tube contained 0.25 cc.; second tube 0.20 cc.; third tube, 0.15 cc.; fourth tube 0.10 cc.; fifth tube 0.05 cc.; sixth tube 0.025 cc. The five following tubes contained the same quantities of antigen and complement as the first five tubes, but they contained no immune serum. These served as the antigen controls. The 12th or last tube contained 0.02 cc. of immune serum and 0.1 cc. of complement but no antigen; this served as the immune serum control.

The volume in each of these twelve tubes was now made up to about 0.3 cc. with normal salt solution, and the rack placed in water bath for half hour at 37°C. After this time sensitized sheep's cells, previously prepared, were added and again placed in the water bath for about ten minutes. The absence of hemolysis in any of the tubes containing immune serum, antigen, complement and sensitized cells proved the presence of complement fixing antibodies in the rabbit's sera tried, providing no hemolysis took place after remaining in the ice-box over night.

Antigens that are somewhat related to one another were interchanged in practically all cases, the same immune serum being employed. Thus, with phaseolin immune serum, both phaseolin

and edestin were employed as antigens. This was done in order to find some possible cases of non-specific binding.

The following is the record of a specific complement-fixation reaction, indicating also the results obtained:

(1) Phaseolin antigen with phaseolin immune serum, prevented hemolysis of sensitized cells, indicating the presence of complement binding antibodies. (2) Phasolin antigen with edestin immune serum did not prevent hemolysis of sensitized cells; i.e., hemolysis was complete. (3) All control tubes wherein, either antigen or immune serum was left out, were also hemolyzed.

In all cases where negative results were obtained double quantities of immune serum were employed and wherever the antigen was not lytic or anticomplementary, double quantities of the antigen also were used, without, however, any indications for complement binding being found. On the other hand, those proteins which gave positive results, produced fixation of complement in all cases in the Wassermann tubes containing 0.10 cc. of antigen (0.10 mgm. of protein), and in some cases, also, in those tubes which contained no more than 0.05 cc. and 0.025 cc. of antigen (0.05 mgm. and 0.025 mgm. of protein).

Immunization of rabbits with egg albumen with a view of calling forth the production of complement-fixing antibodies was also attempted. Two rabbits were employed, one receiving the injections according to method 1, the other according to method 2. But strange as it may seem, particularly in view of the fact that egg albumen is so extensively used in immunization studies, both animals were found dead in their cages. One animal died about twelve hours after the second injection, and the other about one hour after the third injection. It is likely that the quantities employed for injections, although apparently without poisonous effects, in the case of other proteins, were too large in the case of egg albumen.

TABLE 3

Record of results of complement fixation tests employing specific and non-specific immune serum

NUMBER OF EXPERIMENT	DATE	PROTEIN EMPLOYED FOR INJECTION	METHOD OF IMMUNIZATION (TABLE 1)	ANTIGEN EMPLOYED IN COMPLEMENT FIXATION TEST	IMMUNE SERUM EMPLOYED IN COMPLEMENT FIXATION TEST	COMPLEMENT BINDING
	1915					
1	November 10	Phaseolin	No. 1	Phaseolin	Phaseolin	Positive
		Phaseolin	No. 1	Phasolin	Edestin	Negative
2	November 10	Edestin	No. 1	Edestin	Edestin	Positive
		Edestin	No. 1	Edestin	Phaseolin	Negative
3	December 24	Casein	*	Casein	Casein	Negative
		Casein	*	Casein	Lactalbumin	Negative
4	December 24	Lactalbumin	*	Lactalbumin	Lactalbumin	Negative
		Lactalbumin	*	Lactalbumin	Casein	Negative
	1916					
5	January 19	Lactalbumin	No. 1	Lactalbumin	Lactalbumin	Positive
		Lactalbumin	No. 1	Lactalbumin	Casein	Negative
6	January 19	Casein	No. 1	Casein	Casein	Positive
		Casein	No. 1	Casein	Lactalbumin	Negative
7	January 20	Guinea-pig heart	No. 1	Guinea-pig heart	Guinea-pig heart	Negative
		Guinea-pig heart	No. 1	Guinea-pig heart	Guinea-pig muscle	Negative
8	January 20	Guinea-pig muscle	No. 1	Guinea-pig muscle	Guinea-pig muscle	Negative
		Guinea-pig muscle	No. 1	Guinea-pig muscle	Guinea-pig heart	Negative

	1916						
9	February	9	Vaughan's poison from casein	No. 1	Vaughan's poison from casein	Vaughan's poison from casein	Negative
			Vaughan's poison from casein	No. 1	Vaughan's poison from casein	Insoluble residue from Vaughan's poison	Negative
10	February	9	Insoluble residue from Vaughan's poison	No. 1	Insoluble residue from Vaughan's poison	Insoluble residue from Vaughan's poison	Negative
			Insoluble residue from Vaughan's poison	No. 1	Insoluble residue from Vaughan's poison	Vaughan's poison from casein	Negative
11	March	12	Proteose from digestion of racemized casein	No. 2	Proteose from digestion of racemized casein	Proteose from digestion of racemized casein	Negative
			Proteose from digestion of racemized casein	No. 2	Proteose from digestion of racemized casein	Proteose from digestion of gliadin	Negative
12	March	12	Proteose from digestion of gliadin	No. 2	Proteose from digestion of gliadin	Proteose from digestion of gliadin	Negative
			Proteose from digestion of gliadin	No. 2	Proteose from digestion of gliadin	Proteose from digestion of racemized casein	Negative
13	March	12	Proteose from digestion of racemized zein	No. 2	Proteose from digestion of racemized zein	Proteose from digestion of racemized zein	Negative
			Proteose from digestion of racemized zein	No. 2	Proteose from digestion of racemized zein	Proteose from digestion of racemized edestin	Negative
14	March	12	Proteose from digestion of racemized edestin	No. 2	Proteose from digestion of racemized edestin	Proteose from digestion of racemized edestin	Negative
			Proteose from digestion of racemized edestin	No. 2	Proteose from digestion of racemized edestin	Proteose from digestion of racemized zein	Negative

TABLE 3—*Continued*

NUMBER OF EXPERIMENT	DATE	PROTEIN EMPLOYED FOR INJECTION	METHOD OF IMMUNIZATION (TABLE 1)	ANTIGEN EMPLOYED IN COMPLEMENT FIXATION TEST	IMMUNE SERUM EMPLOYED IN COMPLEMENT FIXATION TEST	COMPLEMENT BINDING
	1916					
15	March 15	Racemized zein	No. 1	Racemized zein	Racemized zein	Negative
		Racemized zein	No. 1	Racemized zein	Racemized edestin	Negative
16	March 15	Racemized edestin	No. 1	Racemized edestin	Racemized edestin	Negative
		Racemized edestin	No. 1	Racemized edestin	Racemized zein	Negative
17	March 19	Rabbit liver	No. 1	Rabbit liver	Rabbit liver	Negative
		Rabbit liver	No. 1	Rabbit liver	Rabbit muscle	Negative
18	March 19	Rabbit muscle	No. 1	Rabbit muscle	Rabbit muscle	Negative
		Rabbit muscle	No. 1	Rabbit muscle	Rabbit liver	Negative
19	March 22	Racemized casein	No. 2	Racemized casein	Racemized casein	Negative
		Racemized casein	No. 2	Racemized casein	Racemized egg albumin	Negative
20	March 22	Racemized egg albumin	No. 2	Racemized egg albumin	Racemized egg albumin	Negative
		Racemized egg albumin	No. 2	Racemized egg albumin	Racemized casein	Negative
21	April 10	Phaseolin	No. 2	Phaseolin	Phaseolin	Positive
		Phaseolin	No. 2	Phaseolin	Edestin	Negative
22	April 10	Edestin	No. 2	Edestin	Edestin	Positive
		Edestin	No. 2	Edestin	Phaseolin	Negative
23	April 10	Gelatin	No. 2	Gelatin	Gelatin	Negative

* Methods of immunization not adhered to. Only 5 mgm. of protein injected during each of five intervals (see table 2—experiments 3 and 4).

CONCLUSIONS

A series of protein substances of various origin and chemical constitution were tested with regard to their ability to call forth complement-fixing antibodies in rabbits. Two simple methods of immunization which extended eleven and sixteen days respectively, and which gave positive results with vegetable proteins, were employed throughout the series. The complement fixation test used was the one standardized and employed in the Health Department of New York City.

Two vegetable proteins of high purity—edestin and phaseolin—gave positive complement-fixation reactions. Two animal proteins of relative purity, one of which was a compound protein (casein), and the other a simple protein (lactalbumin), also gave positive complement-fixation reactions. The latter two proteins however, gave negative results at first, as is indicated in table 3 (experiments 3 and 4). The reason for this, was because the quantitative relations of the injections as tabulated in methods 1 and 2, were not abided by. Instead of beginning the injections with solutions containing 50 mgm. of protein and increasing each injection by 25 mgm., solutions were injected which contained not more than 5 mgm. each.

In this regard, therefore, there appears to be an important quantitative difference between the production of anaphylactic antibodies, where a fraction of a milligram often suffices (0.0000001 gram in the case of edestin (3)), and the production of complement-fixing antibodies where much larger quantities of protein are required for immunization. Positive results were obtained with casein and lactalbumin when the experiments were carried out in accordance with method 1.

Gelatin, which lacks cystine, tyrosine, and tryptophane, and has been shown to lack anaphylactogenic properties, gave negative results also in our hands. It is likely that the deficiency of this protein in aromatic radicals, is responsible for its lack of antigenic properties. These radicals, as has been shown by a number of workers (15), appear to be particularly important in immunity reactions. For, as is well known, the mere lack of

amino acids does not prevent a protein from being an antigen. The studies of Wells and Osborne have long demonstrated that such proteins as zein, hordein, and gliadin although lacking one or more amino acid radicals (glycocoll, tryptophane, and lysine), are nevertheless strong antigens.

Four proteoses, as well as Vaughan's soluble poison from casein and the insoluble residue remaining from its preparation, gave negative results. This number is evidently too small to enable one to say that the same would be true in the case of other split proteins. However, in view of the consensus of opinion that split proteins are unable to call forth precipitin and anaphylactic antibodies, it appears likely, by virtue of our negative finding, that split proteins also lack the power of producing complement-fixing antibodies.

Four racemized proteins also gave negative results in our hands. These modified proteins appear to undergo a profound molecular rearrangement during the prolonged alkali digestion. It will be recalled also that they are not split by digestive ferments *in vitro*. Their inability, therefore, to call forth antibody production is, perhaps, to be expected.

Finally, negative results were also obtained with various animal tissues employed in this series. When attempting to immunize animals with animal tissue, several difficulties are encountered. It is virtually impossible to wash them entirely free from extractives, red and white cells, and blood serum. Furthermore we have more than one type of cells to contend with in the tissue itself (parenchymatous, endothelial, connective tissue cells, etc.). These factors would tend to make tissues non-specific antigens. We thus find that the so called antisera produced on injection of various tissues, reported in the literature, contained not only antibodies against the tissues injected, but against leucocytes, red cells, and endothelium as well.

Why the findings in this laboratory were totally negative, is perhaps explained by the fact that the tissues were subjected to boiling temperature during their process of preparation for the injections. It is likely that had they not been boiled, they would have produced in rabbits non-specific immune sera.

SUMMARY

1. Vegetable and animal proteins, injected into rabbits, called forth the production of complement-fixing antibodies in these animals.

2. (a) Gelatin; (b) racemized proteins; (c) proteoses; (d) Vaughan's soluble poison from casein; (e) insoluble residue remaining from the preparation of Vaughan's poison from casein; and (f) animal tissues, did not produce complement fixing antibodies when injected into rabbits.

3. What has been found to be true in the case of other immunity reactions, appears to be true, also, in the case of the complement fixation reaction, namely that *the specificity of the complement fixation reaction depends upon the chemical structure of the protein molecule and if the molecule be split or modified by racemization, its specific complement binding power is lost.*

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REFERENCES

(1) Wells and Osborne: Jour. Inf. Diseases, 1911, **8,** 66; 1913, **12,** 34; 1913, **13,** 103 (White and Avery); 1914, **14,** 364 (Lake, Wells and Osborne), 1914, **14,** 377; 1915, **17,** 259 and 1916, **19,** 183.
(2) Dunbar: Ztschr. f. Immunitätsf., 1019, **4,** 740.
(3) White and Avery: Jour. Inf. Diseases, 1913, **13,** 103.
(4) Lake, Wells and Osborne: Jour. Inf. Diseases, 1914, **14,** 364.
(5) Vaughan: Protein split products in relation to immunity and disease. 1913, p. 99.
(6) Wells: Jour. Inf. Diseases, 1908, **5,** 449.
(7) Kraus: Wein. Klin. Wochensch., 1897 No. 32, 736.
(8) Wells: Jour. Inf. Diseases, 1909, **6,** 506.
(9) Wells and Osborne: Jour. Inf. Diseases, 1915, **17,** 259.
(10) Ten Broeck: Jour. Biol. Chem., 1914, **17,** 369.
(11) Underhill and Hendrix: Jour. Biol. Chem., 1915, **22,** 453.
(12) Bradley and Sansum: Jour. Biol. Chem., 1914, **18,** 497.
(13) Underhill and Hendrix: Jour. Biol. Chem., 1915, **22,** 443, and 465.
(14) Kolmer and Trist: Jour. Inf. Diseases, 1916, **18,** 20.
(15) Wells: Chemical pathology. 2d edition. 1914, 179.